IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket: SOLOMON=2B.2

In re Application of:	)	Conf. No.: 9533
Beka SOLOMON et al	)	Art Unit: 1649
Appln. No.: 10/749,522	)	Examiner: K. A. Ballard
Filed: January 2, 2004	)	Washington, D.C.
For: AGENTS AND COMPOSITIONS AND METHODS UTILIZING SAME USEFUL IN DIAGNOSING ...	)	

DECLARATION OF BEKA SOLOMON

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
2011 South Clark Place
Customer Window, Mail Stop
Crystal Plaza Two, Lobby, Room 1B03
Arlington, VA 22202

Sir:

I, the undersigned Beka Solomon, hereby declare and state as follows:

I am a professor at Tel Aviv University and an inventor of the invention disclosed and claimed in above-identified application. A true and correct copy of my *curriculum vitae* is attached hereto as Exhibit A.

I have been informed that in the prosecution of parent U.S. Application No. 10/162,889, the Examiner has taken the position that it is problematic to predict the *in vivo* efficacy of using a filamentous bacteriophage that displays an

antibody, or an epitope binding fragment thereof, which binds to an epitope of β-amyloid so to inhibit aggregation of β-amyloid or disaggregate β-amyloid plaques, based solely on the *in vitro* performance of such bacteriophage.

In order to prove the *in vivo* efficacy of a filamentous bacteriophage which displays an antibody or epitope binding fragment thereof which binds to an epitope of β-amyloid, in a recognized mouse model of Alzheimer's disease, and thereby confirm the predictions made from the *in vitro* tests reported in the above-identified specification, a study was conducted in part in my laboratory and in part in a laboratory commissioned by my laboratory.

The starting materials used in the study were prepared in my laboratory under my direct supervision. These starting materials were then given a code name and shipped to the contract laboratory. The contract laboratory administered them to transgenic mice which are a well-known model of Alzheimer's disease. After a period of time, the mice were sacrificed and brain sections were prepared by the contract laboratory and returned to my laboratory. Analyses of these brain sections were then conducted in my laboratory under my direct supervision.

The specifics of the experimentation were as follows:

Preparation of Filamentous Bacteriophage Displaying scFv-508F

Filamentous bacteriophage displaying single-chain antibody scFv-508F was prepared in my laboratory using the techniques set forth in the examples of the above-identified patent application. Specifically, DNA encoding scFv-508F was fused to DNA encoding the minor coat gpIII of fd phage to prepare phagemid DNA, which was introduced into *E. coli* K91K cells by transformation and recombinant bacteriophage were isolated from transformants so as to produce bacteriophage displaying scFv-508F. scFv-508F is single chain antibody that binds to β-amyloid, and was prepared as described in the examples of the above-identified patent application. The resulting filamentous bacteriophage product was given the code name "BS-5".

Immunization of hAPP751 Transgenic Mice

My laboratory sponsored a study project to be conducted by JSW-Research, Forschungslabor GmbH, in Graz, Austria (hereinafter JSW). The study director was Dr. Birgit Hutter-Paier. As part of this study, the BS-5 bacteriophage was sent to JSW without advising JSW of its specific composition. JSW was requested to immunize hAPP-transgenic mice with this composition and, after an appropriate time,

sacrifice the mice and prepare brain sections to be returned to my laboratory for analysis.

Submitted herewith is a Declaration of Dr. Hutter-Paier stating what steps were conducted by JSW between the time that they received the BS-5 bacteriophage from my laboratory and the time they returned the brain sections to my laboratory.

As indicated in Dr. Hutter-Paier’s Declaration, the tests were conducted with hAPP751-transgenic mice. These mice are a well-known model for the progressive learning and memory impairment that is a cardinal feature of Alzheimer's disease, as is established in Moran et al, "Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human β-amyloid precursor protein", PNAS USA 92:5341-5345 (1995). They also show age-related β-amyloid deposition. See Higgins et al, "Early Alzheimer's disease-like histopathology increases in frequency with age in mice transgenic for β-APP751" PNAS USA 92:4402-4406 (1995). A copy of each of these publications is submitted herewith.

More specifically, according to Dr. Hutter-Paier, ten hAPP751 transgenic mice (age 10 months) were given 100µl of undiluted BS-5 via intranasal administration. I can confirm that 100µl of undiluted BS-5 corresponds to 10^{11} filamentous phage carrying the 508F-scFv. The mice received a

total of six administrations. The time schedule of the administrations is shown in Figure 1 as attached to Dr. Hutter-Paier's Declaration. As stated in Dr. Hutter-Paier's Declaration, nearly six months after initiation of the immunization, the mice were euthanized with chloroform and immediately decapitated. After brain removal, one hemisphere was post-fixed for 24 hours in 4% paraformaldehyde/PBS (pH 7.4). After 24 hours of post fixation, hemispheres were transferred to PBS (pH 7.4), embedded in paraffin, and later on shipped to my laboratory for analysis. Subsequently, similarly prepared brain sections from untreated hAPP(751) mice were also obtained from JSW for use as controls.

Preparation of Brain Tissue

In my laboratory, the hemispheres received from Dr. Hutter-Paier were fixed in 4% paraformaldehyde for 2 hours, and then kept in 10% formalin saline for 2 days at room temperature. Serial coronal sections (5 µm), in an anterior to posterior direction 250 µm apart from each other, were cut and prepared for histology.

Quantitative Analysis of Amyloid Load

Quantitative analysis of β-amyloid deposits in the hemispheres was performed after thioflavin-S staining. Two well-defined coronal sections at the levels of −1.6 and −3.6

from bregma, respectively, were selected for quantification of the amount of β-amyloid load. Sections were deparaffinized, hydrated and stained first with hematoxylin to quench autofluorescence and then with 1% thioflavin-S for 3 minutes followed by immersion in 1% acetic acid for 20 minutes, washed, cleared and mounted. Images from of the thioflavin-S stained hemisphere-brain sections were captured by a CCD color video camera (ProgRes C14, Jenoptic, Jena, Germany) attached to a Leica DMLB microscope (Leica, Germany) and analyzed with appropriate software (Leica Qwin, Leica, Germany).

The total β-amyloid dense core load in deposits, expressed as a percentage of the area stained with thioflavin-S out of the total area of the sections, is shown in Figure 1 attached hereto. Each of the columns in Figure 1 represents a different one of the ten mice used in the test.

Figure 2 shows the effect of phage scFv on amyloid deposit load. Fig. 2A is a scatter plot of the amyloid deposit in individual treated and control mice. Fig. 2B shows the thioflavin-S stained amyloid deposits in a brain section of a transgenic control mouse, while Fig. 2C shows the thioflavin-S stained brain section of a phage-scFv treated mouse. Figs. 2D and E show olfactory sections of a transgenic control mouse and a phage-scFv treated mouse, respectively, stained with thioflavin-S. Fig. 2F shows a summary of the

average amyloid deposit load in the olfactory sections of transgenic control (n=8) and phage scFv treated mice (n=10), the asterisk denoting $p<0.0001$.

As shown in Figs. 1 and 2A, intranasal treatment of the mice with filamentous bacteriophage displaying scFv-508 resulted in 4 of the 10 treated mice being totally devoid of amyloid aggregation, 4 showed a reduction between 60%-80%, while only 2 showed little effect on β-amyloid aggregation. Brain tissues from mice which contained no amyloid deposits were re-examined for their hAPP gene existence and proved positive (data not shown). The mice in the control group contained amyloid deposit load values ranging from 1.05% to 2.8%. Overall, the treatment resulted in a reduction of about 70% in amyloid deposit load compared to the transgenic untreated animals. The olfactory bulbs were the most affected areas in treated mice, exhibiting an average of 90% reduction in amyloid deposit load (Fig. 2D-F).

The *in vivo* results described herein indicate that filamentous bacteriophage displaying an anti-β-amyloid antibody entered the mouse brains and caused clearance of amyloid deposits in the olfactory systems and in other brain regions, thereby establishing the efficacy of such administration in terms of inhibiting β-amyloid aggregation

and causing a significant amount of amyloid deposit disaggregation in a significant number of test mice.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

7/6/06
Date

Beka Solomon
Beka SOLOMON

FIGURE 1

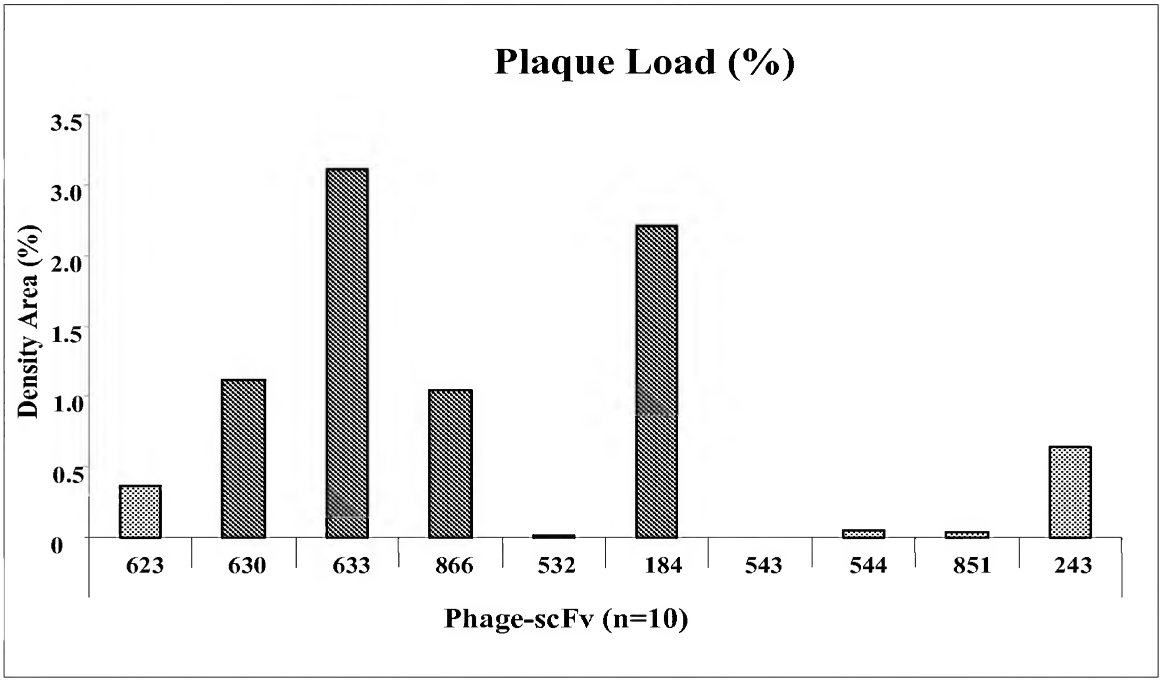
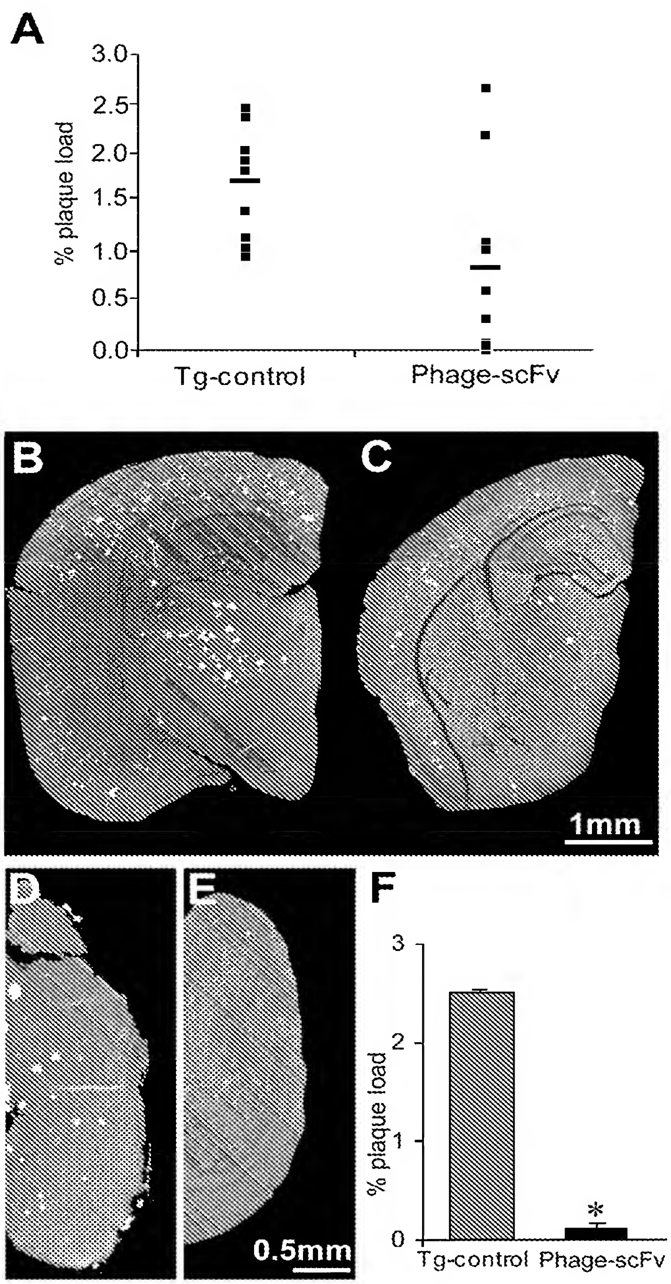

Plaque Load (%)
Density Area (%)
3.5
3.0
2.0
1.5
1.0
0.5
0
623
630
633
866
532
184
543
544
851
243
Phage-scFv (n=10)

FIGURE 2


A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
% plaque load
Tg-control
Phage-scFv
B
C
1mm
D
E
0.5mm
F
3
2
1
0
% plaque load
*
Tg-control
Phage-scFv

CURRICULUM VITAE

Professor Beka Solomon	POSITION TITLE **Head Neuroimmunology Laboratory**

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Polytechnic Institute, Romania	B.Sc /M.Sc	1959-1964	Chemical Engineering, Biophysics/
Weizmann Institute of Science, Israel	Ph.D.	1977	Biochemistry
University of Teheran, Iran	Postgraduate	1977	
Weizmann Institute of Science, Israel	Postgraduate	1978-1979	Insulin receptors
Royal Postgraduate Medical School, London, UK	Postgraduate	1985	Monoclonal antibodies
University of Warwick, Coventry, UK	Postgraduate	1985	Molecular Biology
University of Baltimore, Maryland, USA	Postgraduate	1993	

Positions and Employment

1987-1988 Harvard Medical School and Brigham and Women's Hospital, Biophysics/Biochemistry
1989 - 1996 Tel-Aviv University, Senior Lecturer, Molecular Microbiology - Biotechnology
1996-2002 Tel-Aviv University, Associate Professor, Microbiology - Biotechnology
2002-present Tel-Aviv University, Professor, Microbiology - Biotechnology
2003 Tel-Aviv University, Incumbent of the Chair for Biotechnology of Neurodegenerative Diseases

Other Experience and Professional Memberships

1984 Israel Biochemistry Society
1986 Australian Biotechnology Society
1989 Society for Molecular Recognition
1994 New York Academy of Science
2003 Society of Neuroscience
2000-present Associate Editor of Drugs of Today
2005 Editorial Advisory Board Member – Patent Reviews on CNS Drug Discovery
2005 Editorial Board – Neurodegenerative Disorders

Honors

2002-2004 *In vivo* targeting of beta-amyloid plaques towards diagnosis of Alzheimer's Disease. Alzheimer's Association. Zenith Fellows Award
2005 "Development of a New Immunological Approach for the Treatment of Alzheimer's Disease" Dana Foundation

Selected peer-reviewed publications

EXHIBIT A

Solomon, B., Koppel, R., Hanan, E. and Katzav, T. Monoclonal antibodies inhibit *in vitro* fibrillar aggregation of the Alzheimer's β-amyloid peptide. *Proc.Nat.Acad.Sci. USA* 93 (1) (1996) 452-455.
Solomon, B., Koppel, R., Frankel, D. and Hanan-Aharon, E. Disaggregation of Alzheimer β-amyloid by site-directed mAb. *Proc. Natl. Acad. Sci. USA* 94 (1997) 4109-4112.
Frenkel, D., Balass, M. and **Solomon, B.** N-terminal EFRH sequence of Alzheimer's β-amyloid peptide represents the epitope of its anti-aggregating antibodies. *J. Neuroimmunology* 88 (1998) 85-90.
Frenkel, D. Balass, M., Katchalski-Katzir, E. and **Solomon, B.** High affinity binding of monoclonal antibodies to the sequential epitope EFRH of β-amyloid peptide is essential for modulation of fibrillar aggregation. *J. Neuroimmunology* 95 (1999) 136-142.
Frenkel, D., **Solomon, B.** and Benhar, I. Modulation of Alzheimer's β-amyloid neurotoxicity by site-directed single-chain antibody. *J. Neuroimmunol.* 106 (2000) 23-31.
Frenkel, D., Kariv, N. and **Solomon, B.** Generation of auto-antibodies towards Alzheimer's disease vaccination. *Vaccine* 19, 2615-2619. (2001).
Frenkel, D., Katz, O. and **Solomon, B.** Immunization against Alzheimer's β-amyloid plaques via EFRH administration. *Proc. Natl. Acad. Sci. USA* (2000) 97. 11455-11459.
Hanan, E., Goren., Azhkenasi, M. and **Solomon, B.** Immunomodulation of neurotoxicity of prion peptide 106-126. *Biochem. Biophys. Res. Com.* (2001) 280, 115-120.
Solomon, B., Koppel, R. and Jossiphov. J. Calmodulin and aluminium immunoreactivity in Alzheimer's disease. *Brain Research Bull.* 55, (2001) 253-256 .
Solomon, B. and R. Koppel. IgM detection via selective recognition by mannose-binding protein. *J. Biochem. Biophys. Meth.* 49 (2001) 641-647.
Levy, Y., Hanan, E., **Solomon, B.** and Becker, O.M. The helix-coil transition of PrP 106-126: a molecular dynamic study. *Proteins: Structure, Function and Genetics* 45 (2001) 382-396.

Govorko, D., Cohen, G. and **Solomon, B.** Single chain antibody against the common epitope of mutant p53: isolation and intracytosolic expression in mammalian cells. *J. Immunol. Meth.* 258 (2001) 169-181.
Hanan, E., Priola, S.A. and **Solomon, B.** Anti-aggregating antibody raised against human PrP 106-126 recognizes pathological and normal isoforms of the whole prion protein. *Cellular and Molec. Neurobiology.* (2001). 21(6):693-703.
Frenkel, D., and **Solomon, B.** Filamentous phage as vector-mediated antibody delivery to the brain. *Proc. Natl. Acad. Sc. USA.* (2002) 16;99(8):5675-9.
Pan, W., **Solomon, B.**, Maness, L.M., Kastin, A.J. Antibodies to beta-amyloid decrease the blood-to-brain transfer of beta-amyloid peptide. *Exp. Biol Med (Maywood)* (2002) 227(8): 609-15.
Solomon, B. and Frenkel, D. Generation and brain delivery of anti-aggregating antibodies against β-amyloid plaques using phage display technology. *J. Neural Transmission.* (2002) 62: 319-323.
Solomon, B. Protection molecules in Alzheimer's disease: Therapeutic antibodies. (2002) *Drug News Perspectives.* 15(7): 410-416.
Solomon, B. Immunological Approaches as Therapy for Alzheimer's Disease. *Exp. Opp. Biol. Therapy.* (2002) 2 (8): 907-917.
Frenkel D, Dewachter I, Van Leuven F, **Solomon B.** Reduction of beta-amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization. *Vaccine* (2003) 7;21(11-12):1060-5.
Arbel M., Lavie, V. and **Solomon, B.** Generation of antibodies against prion protein in wild-type mice via helix 1 peptide immunization. *J. Neuroimmunol.* (2003), 144:38-45.
Frenkel D, Dori M, **Solomon B.** Generation of anti-beta-amyloid antibodies via phage display technology. *Vaccine* (2004) 22(19):2505-8.
Lavie V, Becker M, Cohen-Kupiec R, Yacoby I, Koppel R, Wedenig M, Hutter-Paier B, **Solomon B.** EFRH-Phage immunization of Alzheimer's disease animal model improves behavioral performance in Morris Water Maze trials. *J Molec Neurosc* (2004) 24:105-113.
Rebe S and **Solomon B.** Deglycosylation of anti-beta amyloid antibodies inhibits microglia activation in VB-2 cellular model. *Am J Alz Dis and Other Dementias* (2005) 20 (5):303-313.
Orgad S, Goldfinger N, Cohen G, Rotter V and **Solomon B.** Single chain antibody against the common epitope of mutant p53 restores wild-type activity to mutant p53 protein. *FEBS* (2005) 579:5609-5615.
Arbel M, Yacobi I, **Solomon B.** Inhibition of amyloid precursor protein processing by beta-secretase through site-directed antibodies. *Proc Natl Acad Sci USA* (2005) 10:102(21):7718-23.

Invited Papers

Solomon, B. and Frenkel, D. Vaccination towards prevention and treatment of Alzheimer's disease. *Drugs of Today (*2000) 36, 655-663.
Solomon, B. Immunotherapeutic strategies towards prevention and treatment of Alzheimer's disease. *DNA and Cell Biology.* (2001) (11):697-703.
Solomon, B. Towards Alzheimer's disease vaccination. *Mini-Reviews in Medicinal Chemistry.* (2002) 2(1): 85-92.
Solomon, B. Anti-aggregating antibodies, a new approach towards treatment of conformational diseases. *Current Topics in Medicinal Chemistry.* (2002) 9(19): 1737-49.
Solomon, B. Immunological concept in the treatment of Alzheimer's disease. *Drug Development Research.* Review (2002) 56(2): 163-167.
Solomon B. Alzheimer's Disease and Immunotherapy. *Current Alzheimer* Research. (2004) 1:149-163.
Solomon B. Generation of anti-beta amyloid antibodies via phage display technology towards Alzheimer's disease vaccination. *Vaccine* (2005) 18:2327-30.
Solomon, B. Peptide vaccine for Alzheimer's disease. *Handbook of Peptides* (2005) Accepted for publication.
Solomon, B. *In vivo* targeting of amyloid plaques via intranasal administration of phage anti-β-amyloid antibodies. *Proceedings of ADPD Conference* 2005.
Solomon, B. Alzheimer's disease immunotherapy: from *in vitro* amyloid immunomodulation to *in vivo* vaccination. *Alzheimer Anniversary Issue* (2005).

Patents

Title	Application Date	Application No	Country	Patent No
PREVENTION OF PROTEIN AGGREGATION	16/12/1994	08/358,786	U.S.A	5,688,651
PREVENTION OF PROTEIN AGGREGATION	16/11/1999	09/441,140	U.S.A	
SINGLE CHAIN ANTIBODY AGAINST MUTANT P53	16/03/2000	09/526,738	U.S.A	6,630,584
SINGLE CHAIN ANTIBODY AGAINST MUTANT P53	20/09/2002	10/247,488 PCT/IL01/00225 1914142.3	U.S.A PCT TREATY EUROPE	
AGENTS AND COPMPOSITIONS AND METHODS UTILIZING SAME USEFUL IN DIAGNOSING AND/OR TREATING OR PREVENTING PLAQUE FORMING DISEASES (BRAIN DELIVERY OF THERAPEUTIC ANTIBODIES AGAINST AMYLOIDOGENIC DISEASES)	31/08/2000	954883.5 2001-522381 67232/00 PCT/IL00/00518 11/073,526 2106123.5 2,349,434 142948	EUROPE JAPAN Australia PCT TREATY U.S.A HONG KONG CANADA Israeli	
BACTERIOPHAGE DISPLAYING A BETA EPITOPES AND METHOD OF USE	31/08/2000	09/830,954	U.S.A	6,919,075
FILAMENTOUS BACTERIOPHAGE DISPLAYING AN B-AMYLOID EPITOPE	29/12/1999	09/473,653	U.S.A	6,703,015
IMMUNIZATION AGAINST AMYLOID PLAQUES USING DISPLAY TECHNOLOGY	15/07/2003	10/618,856	U.S.A	
MODULATION OF NEUROTOXICITY OF PRION PEPTIDE 106-126 BY MONOCLONAL ANTIBODIES	02/01/2004	10/749,522	U.S.A	
IMMUNIZING COMPOSITION AND MEHTOD FOR INDUCING AN IMMUNE RESPONSE AGAINST THE B-SECRETED CLEAVAGE SITE OF AMYLOID PRECURSOR PROTEIN	04/03/2003	10/506,665 3744143.3 2003-574670 163812 2,477,675	U.S.A Europe JAPAN ISRAEL Canada	

PREVENTION OF BRAIN INFLAMMATION AS A RESULT OF INDUCED AUTOIMMUNE RESPONSE	14/04/2003	10/510,820	U.S.A
METHODS AND COMPOSITIONS FOR TREATING A PLAQUE-FORMING DISEASE	11/03/2003	10/384,788 2721440.2	U.S.A EUROPE
ANTIGENIC PRODUCT DISPLAYING MULTIPLE COPIES OF AN EPITOPE OF A DEPOSIT-FORMING POLYPEPTIDE INVOLVED IN PLAQUE- FORMING DISEASES AND METHODS OF USING SAME	20/06/2002	10/481,642 PCT/US02/19567 2,450,783 2737552.6	U.S.A PCT CANADA EUROPE
METHOD OF PASSIVE IMMUNIZATION AGAINST DISEASE OR DISORDER CHARACTERIZED BY AMYLOID AGGREGATION WITH DIMINISHED RISK OF NEUROINFLAMMATION	06/06/2005	PCT/US05/19617	PCT TREATY

CC

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5341–5345, June 1995
Medical Sciences

Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human β-amyloid precursor protein

(Alzheimer disease/learning)

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Communicated by Seymour S. Kety, National Institutes of Health, Bethesda, MD, March 2, 1995 (received for review December 16, 1994)

ABSTRACT **The β-amyloid precursor protein (β-APP), from which the β-A4 peptide is derived, is considered to be central to the pathogenesis of Alzheimer disease (AD). Transgenic mice expressing the 751-amino acid isoform of human β-APP (β-APP$_{751}$) have been shown to develop early AD-like histopathology with diffuse deposits of β-A4 and aberrant tau protein expression in the brain, particularly in the hippocampus, cortex, and amygdala. We now report that β-APP$_{751}$ transgenic mice exhibit age-dependent deficits in spatial learning in a water-maze task and in spontaneous alternation in a Y maze. These deficits were mild or absent in 6-month-old transgenic mice but were severe in 12-month-old transgenic mice compared to age-matched wild-type control mice. No other behavioral abnormalities were observed. These mice therefore model the progressive learning and memory impairment that is a cardinal feature of AD. These results provide evidence for a relationship between abnormal expression of β-APP and cognitive impairments.**

The neuronal degeneration and ensuing dementia associated with Alzheimer disease (AD) is thought to be related to the accumulation of extracellular deposits of the peptide β-amyloid (β-A4). These deposits form senile plaques in the cortex and hippocampus that are characteristic of the disease. Several lines of evidence suggest that the β-A4 fragment, which is derived from a larger β-amyloid precursor protein (β-APP), is central to the pathogenesis of AD. For example, specific mutations in the β-APP gene, which is located on chromosome 21, have been identified in patients with the familial form of AD (1–3). Furthermore, there is a high incidence of AD in patients with trisomy 21 (Down syndrome) who carry an extra copy of this gene (4, 5). Detailed examination of the precise role that β-A4 plays in the pathology of AD and the development of an appropriate therapy have been hampered by the lack of a suitable animal model. Very few animals naturally develop β-A4 deposits with age, and rodents rarely display such deposits. One experimental approach that has recently been explored is the development of transgenic animals that express the human β-APP gene or some portion of it. There are at least six mRNAs produced by splice variants of the β-APP gene (6). Four of these isoforms (695, 714, 751, and 770 amino acids long) contain the β-A4 segment and two of these, the 770- and 751-amino acid isoforms, are homologous to the Kunitz family of serine proteinase inhibitors (7, 8). Transgenic mice have been created in which expression of the human 751-amino acid isoform of β-APP (β-APP$_{751}$) coupled to a neuron-specific promoter leads to diffuse deposits of β-A4 and increased tau protein immunoreactivity, particularly in the hippocampus, cortex, and amygdala, areas of the brain that are associated with cognitive function (9–11). The following study investigated the performance of transgenic mice expressing human β-APP$_{751}$ from two age groups in a variety of general behavioral and cognitive tasks.

MATERIALS AND METHODS

Animals. Female transgenic mice that were homozygous for the transgene of human β-APP$_{751}$ cDNA under the control of a neuron-specific enolase promoter were used. Young mice were 5–6 months old (n = 12 wild type; n = 10 transgenic) and aged mice were 9–12 months old (n = 12 per group) at the start of testing. They were housed in microisolated cages in groups of 2–4 mice, in a temperature (20 ± 2°C)-controlled environment. All mice were kept on a 12-h light/12-h dark cycle and allowed free access to food and water in their cages. In the aged group, one mouse died during the test period and was excluded from the entire study; another mouse was excluded from the water maze study as it had difficulty swimming.

All experiments were carried out blind.

General Behavioral Tests. *String test.* The apparatus was a length of string (50 cm) pulled taut between two vertical supports and elevated 40 cm from a flat surface. The mouse was placed on the string at a point midway between the supports and was rated by the experimenter according to the following system: 0, falls off; 1, hangs onto string by two forepaws; 2, as for 1, but attempts to climb onto string; 3, hangs onto string by two forepaws plus one or both hindpaws; 4, hangs onto string by all four paws plus tail wrapped around string; 5, escape (where mouse was able to work its way to one of the supports).

Rotarod. A standard rotarod apparatus was used consisting of a motor-driven rotating cylinder (3 cm in diameter and 12 cm wide; 26 rotations per min) upon which the mice were placed individually. Each time a mouse fell off it was replaced on the rotarod. The number of times the animal fell off in 60 s was counted.

General activity. This was measured by placing the mice individually into photocell cages (40 cm × 25 cm × 15 cm). Horizontal infrared beams were located perpendicularly to the long axis of the cage every 2.5 cm and 2 cm above the base of the cage. The number of times the photobeams were broken was automatically recorded every hour over a 23-h period from 1200 to 1100 hours.

Body temperature. The core body temperature of the mice was taken five times throughout the day between 0815 and 1800 by using a thermistor probe inserted 2 cm into the rectum.

Elevated plus-maze test. This was carried out essentially as described (12). The apparatus was an elevated plus-maze consisting of two open arms (50 × 10 cm) and two closed arms (as open arms but with 40 cm high side and end walls) raised 50 cm off the floor in a room lit with a red light. Mice were placed in the center of the plus-maze facing an open arm and observed for 5 min via a camera and black and white monitor

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Abbreviations: β-APP, β-amyloid precursor protein; β-APP$_{751}$, 751-amino acid isoform of β-APP; AD, Alzheimer disease; β-A4, β-amyloid.
†To whom reprint requests should be addressed.

by an observer located in an adjacent room. A variety of measures were taken in real time and stored directly on a computer using the OBSERVER program (Noldus Information Technology, Wageningen, The Netherlands). The parameters measured were open-arm entries and time spent on open arms, frequency of stretched attend posture, rearing, and grooming. Open-arm entries and time spent on open arms are expressed as percentages of combined open- and closed-arm entries and combined open- and closed-arm time, respectively.

Behavioral despair. The mouse was placed in a 5-liter container filled with water to a depth of 30 cm. The amount of time spent actively swimming and moving during a 4-min observation period was measured by direct observation.

Spontaneous Alternation in a Y Maze. The apparatus was a symmetrical Y maze, each arm measured 50 × 10 cm, with 40-cm high side walls. Mice were placed in the maze and allowed to freely explore for 5 min. Arms were arbitrarily labeled A, B, and C, and the sequence of arm entries was used to measure alternation behavior: percent alternation is the number of alternation sequences (i.e., ABC, ACB, CAB, etc.) divided by the number of alternation opportunities (equivalent to the total number of arm entries minus 2). An unbiased alternation score (i.e., corrected for left and right going alternations) and a measure of position bias (egocentric turning) were also calculated (13). Two 6- and one 12-month-old wild-type mice and one 12-month-old transgenic mouse were excluded as they made too few entries (<12) to accurately determine alternation scores.

Water Maze. The water maze was an 80-cm-diameter black cylindrical pool filled with water heated to 26°C that contained a 6-cm-diameter cylindrical wire mesh platform hidden 0.5 cm below the surface of the water. This was located in an experimental room rich in environmental cues, such as a window, air conditioning conduits, and tables. An overhead camera connected to a computerized movement tracking system (Viewpoint, Lyon, France) recorded the swim paths of the mice in the maze and their latency to locate the platform.

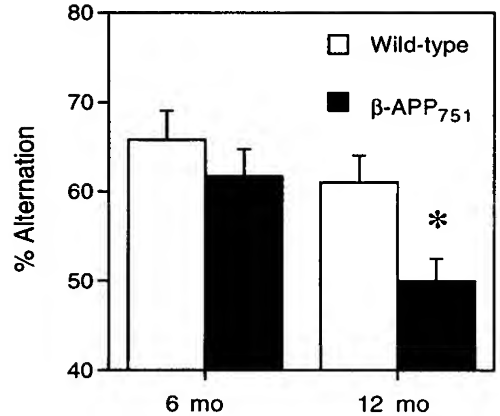

FIG. 1. Spontaneous alternation in a Y maze. The values shown represent the percentage alternation during a 5-min period (mean ± SEM). Differences in percentage alternation between wild-type and transgenic mice were compared by using the Mann–Whitney U test (the asterisk denotes a significant difference at $P < 0.05$).

After a 60-s habituation trial to verify their ability to swim, the mice were given 18 training trials during which their latency to find the hidden platform was measured up to a maximum of 60 s. If the mouse failed to locate the platform within this time, it was placed on the platform by the experimenter for 10 s. Each mouse received two trials a day, with a probe trial after each block of six trials. A probe trial consisted of a 60-s trial during which there was no platform present in the maze and during which its search pattern was analyzed. The percentage of time spent searching the quadrant of the maze that formerly contained the platform was calculated and compared with the

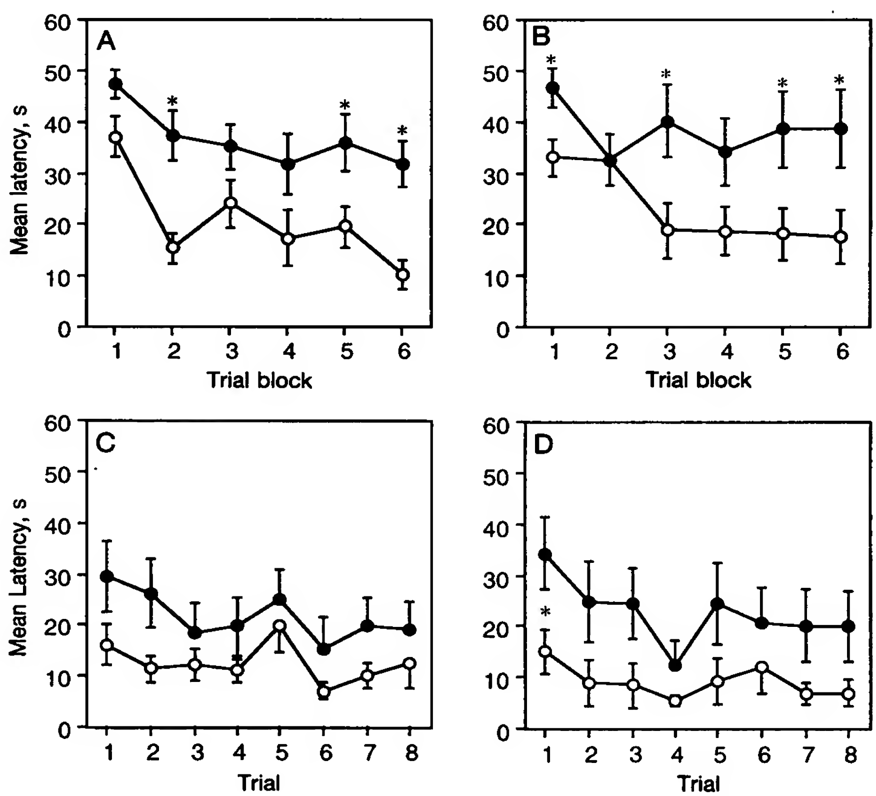

FIG. 2. Latencies (mean ± SEM) to find a platform in a water maze. (*A* and *B*) Submerged platform. (*C* and *D*) Visible platform. (*A* and *C*) Six months old. (*B* and *D*) Twelve months old. Open symbols, values for wild-type mice (mean ± SEM; $n = 12$); solid symbols, values for transgenic mice [n (6 months old) = 10, n (12 months old) = 12]. Significant differences between groups of mice are indicated by asterisks ($P < 0.05$, t test following significant ANOVA).

percentage of time spent searching the other quadrants of the maze. The number of times the mice crossed the exact former platform location (annulus entries) and the corresponding zones in the other quadrants was also recorded.

In the visible platform version of the task, a white platform was raised above the surface of the water and a flag was placed in the middle to make it more visible. The position of the platform was different for each of the eight trials. Latency to locate the platform was recorded up to a maximum of 60 s.

RESULTS

General Behavioral Tests. There were no significant differences between transgenic and wild-type control mice in either 6- or 12-month-old groups in the string test of muscle strength, the rotarod test of motor coordination, the circadian variation in body temperature, the plus maze test of anxiety, or the behavioral despair test. The circadian pattern of spontaneous motor activity was similar between wild-type and transgenic mice in both age groups. However, over the 24-h test period both 12-month-old ($F_{1,22} = 6.77$; $P < 0.01$) and 6-month-old ($F_{1,20} = 10.05$; $P < 0.01$) transgenic mice showed lower activity than wild-type mice. This decreased activity was seen only during the dark phase.

Spontaneous Alternation in a Y Maze. There was no significant difference between transgenic and wild-type groups in spontaneous alternation scores in 6-month-old mice (Fig. 1). In the 12-month-old groups, transgenic mice alternated significantly less than wild-type control mice ($P < 0.05$, Mann–Whitney U test). Analysis of the data according to the method of McFarland (13), which corrects for possible turning bias, did not significantly change the alternation scores. In addition, there was no significant difference between wild-type and transgenic mice of either age group in the turning bias measure (6-month-old wild type, 67.7 ± 2.7; 6-month-old transgenic, 63.1 ± 3.3; 12-month-old wild type, 64.4 ± 2.0; 12-month-old transgenic, 63.2 ± 3.1).

Water-Maze Acquisition Latencies (Hidden Platform). Both the 6-month-old transgenic mice ($F_{1,22} = 12.31$; $P < 0.01$) and the 12-month-old transgenic mice ($F_{1,20} = 6.19$; $P < 0.05$) showed significantly higher latency values than wild-type controls (Fig. 2 *A* and *B*). However, the latency of wild-type and transgenic groups of both ages decreased similarly with training as suggested by the lack of significant trial block–genotype interaction in either 6-month-old ($F_{5,1} = 1.0$; nonsignificant) or 12-month-old ($F_{5,1} = 1.9$; nonsignificant) groups.

Water-Maze Acquisition Latencies (Visible Platform). There was no overall significant difference between transgenic and wild-type mice in latencies to locate the visible platform in either 6-month-old ($F_{1,7} = 2.5$; nonsignificant) or 12-month-old ($F_{1,7} = 3.7$; nonsignificant) mice (Fig. 2 *C* and *D*).

Water-Maze Probe Trials. Differences in time spent in each quadrant were analyzed for each probe trial by using within-group analyses of variance. During the first probe trial 6-month-old transgenic mice searched the training quadrant significantly more than other quadrants ($F_{3,33} = 3.25$; $P < 0.05$), although this was not the case for wild-type controls ($F_{3,33} = 2.38$; nonsignificant). However, by probe trials 2 ($F_{3,33} = 3.9$; $P < 0.05$) and 3 ($F_{3,33} = 22.56$; $P < 0.0001$), wild-type mice selectively searched the training quadrant. In the transgenic group, this selective searching failed to reach significance on probe trial 2 ($F_{3,33} = 2.22$; nonsignificant) but was again evident by probe trial 3 ($F_{3,33} = 5.17$; $P < 0.01$).

In 12-month-old mice, no significant difference in percent search time per quadrant was evident during the first probe trial for either group of mice (Fig. 3). However, during the second and third probe trials the wild-type mice searched the training quadrant significantly more than other quadrants (probe trial 1, $F_{3,33} = 1.52$, nonsignificant; probe trial 2, $F_{3,33} = 8.8$, $P < 0.001$; probe trial 3, $F_{3,33} = 5.14$, $P < 0.01$). No significant differences were apparent in this measure for the transgenic mice (probe trial 1, $F_{3,27} = 1.32$, nonsignificant; probe trial 2, $F_{3,27} = 1.91$, nonsignificant; probe trial 3, $F_{3,27} = 0.28$, nonsignificant). The absence of a selective search pattern in these mice compared to wild-type controls is illustrated in Fig. 4.

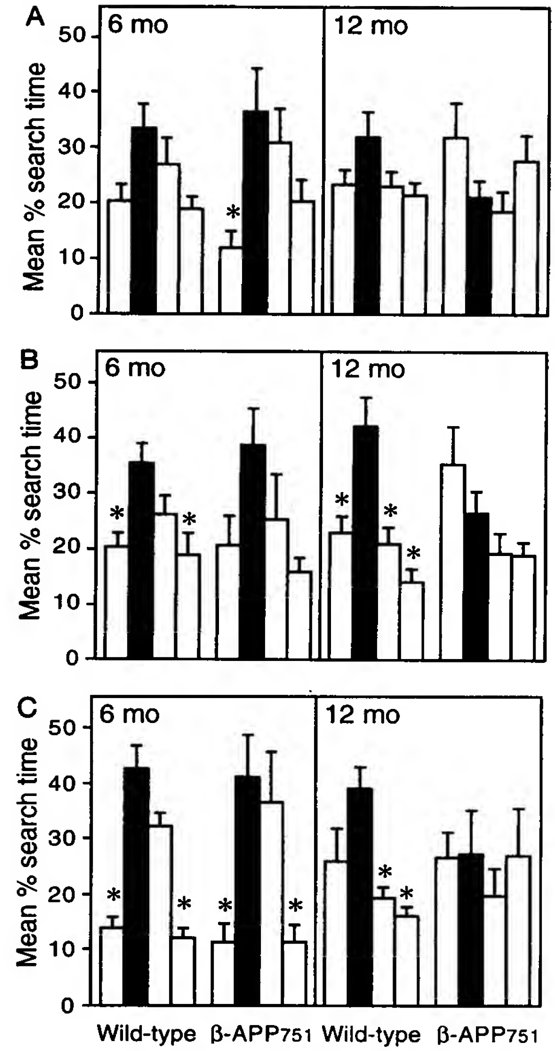

FIG. 3. Mean percent search times per maze quadrant during three probe trials in a water maze. The four bars represent the data for the four quadrants in the following order: adjacent left, training (solid bars), adjacent right, and opposite. Each probe trial consisted of a 60-s trial during which the platform was removed from the maze. Asterisks indicate a significant difference ($P < 0.05$) compared to the training quadrant (t test following a significant ANOVA). (*A*) Probe trial 1. (*B*) Probe trial 2. (*C*) Probe trial 3. mo, Months old.

Water-Maze Annulus Entries. There were no significant differences in the number of annulus entries made by 6-month-old wild-type and transgenic mice (Table 1). In 12-month-old mice, the transgenic group made significantly fewer annulus entries than wild type in probe trials 1 and 2 ($P < 0.05$, t-test).

DISCUSSION

The results of the present study demonstrate that mice expressing human β-APP_{751} have learning and memory deficits. Learning deficits were seen in the water maze, a test of spatial learning, and a memory deficit was indicated by impaired spontaneous alternation, a task that involves working memory. These deficits were seen in 12-month-old but not 6-month-old transgenic mice, demonstrating that these effects are age-dependent. This learning and memory impairment occurred in

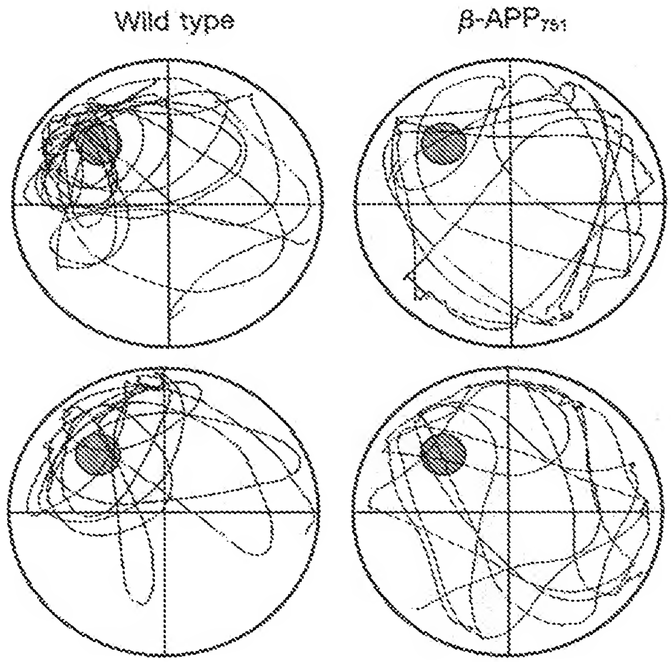

FIG. 4. Typical examples of search patterns recorded during probe trials 2 and 3 for 12-month-old wild-type and transgenic mice. The former platform location is indicated by the shaded circle in the upper left quadrant of the maze. Twelve-month-old wild-type mice concentrated their searching around the former platform location site, and transgenic mice of the same age swam randomly around the maze clearly showing no preference for any one area of the maze.

the absence of generalized behavioral deficits or marked changes in activity or anxiety in transgenic groups.

In the water maze, transgenic mice of both age groups had longer latencies to locate the hidden platform than did wild-type mice. However, the 6-month-old transgenic group showed a decrease in time taken to find the platform with training, indicative of retarded rather than abolished task acquisition. The 12-month-old transgenic group did not show such a decrease with training, strongly suggesting an acquisition deficit in the older transgenic group. Latency can be confounded by other factors such as swim speed and differences in search strategy. Probe trials were, therefore, used to assess spatial strategy. The probe trial data showed that 6-month-old transgenic mice displayed a similar degree of spatial learning to wild-type mice of the same age and 12-month-old wild-type mice. On the second and third probe trials, these groups of mice were selectively searching the former training quadrant of the maze and crossing the exact former platform location more than comparable areas in other maze quadrants. In contrast, the 12-month-old transgenic mice did not show this selective search strategy. It was noted during training and probe trials that some of the 12-month-old transgenic mice showed a tendency to confine their searching to the perimeter of the maze, circling the edge of the maze and scratching at the walls. This behavior is unlikely to represent a lack of motivation to find the platform as all the mice climbed onto and remained on the platform when they located it or when placed on it by the experimenter, and they were proficient at the visual platform task. Interestingly, this type of aberrant swimming behavior has previously been reported in rodents after hippocampal lesion and anticholinergic drug treatment (14–16).

Table 1. Annulus entries during probe trials

Probe trial	6 months old		12 months old	
	Wild type	Transgenic	Wild type	Transgenic
1	2.42 ± 0.62	1.08 ± 0.34	3.33 ± 0.57	1.50 ± 0.64*
2	2.83 ± 0.63	1.50 ± 0.34	5.00 ± 0.71	1.60 ± 0.69*
3	2.83 ± 0.76	2.00 ± 0.59	4.00 ± 0.59	2.50 ± 0.95

All values are the mean ± SEM. The *n* numbers are as in Fig. 2.
*Significant difference compared to the relevant wild-type group by a Student's *t* test.

Spontaneous alternation behavior was similar to controls in 6-month-old transgenic mice and disrupted to chance levels in the 12-month-old transgenic group. Control mice and 6-month-old transgenic mice showed an orderly pattern of exploration of the maze, as indicated by the high level of spontaneous alternation. In contrast, 12-month-old transgenic mice showed random exploration of the Y maze as demonstrated by the low alternation score. The alternation scores obtained in either transgenic or wild-type mice were not due to repetitive turning to the left or right side (which would produce artificially high alternation scores), as no differences in turning bias were observed in any group. Spontaneous alternation has been suggested to require a rudimentary form of working memory (17–19). This effect, like the spatial learning deficit detected in the water maze, was age-dependent as no deficit in spontaneous alternation was detected in 6-month-old transgenic mice.

Neither 6-month-old nor 12-month-old transgenic mice showed any changes in string test, rotarod, plus-maze, or circadian variation in spontaneous activity, showing that they did not have gross motor, physiological, or behavioral impairments that may have confounded interpretation of the learning tasks. A decrease in nocturnal activity was found in transgenic mice of both age groups, but this was unlikely to have affected the results from learning experiments as it was seen in both 6-month-old and 12-month-old mice while learning deficits were seen only in the 12-month-old transgenic group.

Previous studies have demonstrated that this pedigree of β-APP_{751} transgenic mice have an age-related increase in deposits of β-A4, particularly in the cortex and the hippocampus, and abnormal tau protein (20). It is therefore significant that the cognitive deficit we demonstrate here also increases with age. Further studies will be required to demonstrate whether or not a direct causal relationship exists between β-A4 deposition and/or aberrant tau and the cognitive deficits we have reported and to characterize the underlying mechanism(s) involved.

One possible implication of the present results is that abnormal expression of Kunitz proteinase inhibitor-containing β-APP isoforms is important for the development of learning impairment and AD-like histopathology. Previous studies have reported that transgenic mice expressing the 695-amino acid isoform of β-APP, which does not contain the Kunitz proteinase inhibitor domain, do not develop histopathological abnormalities (9, 10) nor do they show a deficit during probe trials in a water-maze task, although a slight increase in acquisition latencies has been reported (21). This contrasts with β-APP_{751} mice, which develop histopathological abnormalities for both β-amyloid and tau protein (20) and display marked disruption of learning and memory. It will be of interest to compare these data with the behavioral phenotype, if any, of the transgenic mouse recently reported by Games *et al.* (22) that have robust β-amyloid pathology but no tau histopathology.

In conclusion, our results demonstrate that mice expressing human β-APP_{751} develop a learning deficit between 6 and 12 months of age and that this occurs in the absence of generalized neurological deficits or marked changes in activity or anxiety. This model will, therefore, be a valuable tool in advancing our understanding of the relationship between β-APP and the cognitive deficits found in AD.

1. Goate, A. M. (1991) *Nature (London)* **349,** 704–706.
2. Chartier-Harlin, M. C., Crawford, F. & Houlden, H. (1991) *Nature (London)* **353,** 844–846.

3. Murrell, J., Farlow, M., Ghetti, B. & Benson, M. D. (1991) *Science* **254,** 97–99.
4. Mann, D. M. A. (1988) *Mech. Ageing Dev.* **43,** 99–136.
5. Mann, D. M. A., Jones, D., Prinja, D. & Purkiss, M. S. (1990) *Acta Neuropathol.* **80,** 318–327.
6. Joachim, C. L. & Selkoe, D. J. (1992) *Alzheimer Dis. Assoc. Disord.* **6,** 7–34.
7. Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F. & Cordell, B. (1988) *Nature (London)* **331,** 525–527.
8. Tanzi, R. E., McClatchey, A. I., Lamperty, E. D., Villa-Komaroff, L., Gusella, G. F. & Neve, R. L. (1988) *Nature (London)* **331,** 528–530.
9. Quon, D., Wang, Y., Catalano, R., Marian Scardina, J., Murakami, K. & Cordell, B. (1988) *Nature (London)* **352,** 239–241.
10. Higgins, L. S., Catalano, R., Quon, D. & Cordell, B. (1993) *Ann. N.Y. Acad. Sci.* **695,** 224–227.
11. Higgins, L. S., Holtzman, D. M., Rabin, J., Mobley, W. C. & Cordell, B. (1994) *Ann. Neurol.* **35,** 598–607.
12. Moser, P. C. (1991) *Psychopharmacology* **99,** 48–53.
13. McFarland, D. J. (1989) *Pharmacol. Biochem. Behav.* **32,** 723–726.
14. Whishaw, I. Q. & Tomie, J.-A. (1987) *Behav. Neurosci.* **101,** 603–616.
15. Whishaw, I. Q., Mittleman, G., Bunch, S. T. & Dunnett, S. B. (1987) *Behav. Brain Res.* **24,** 125–138.
16. Schenk, F. & Morris, R. G. (1985) *Exp. Brain Res.* **58,** 11–28.
17. Still, A. W. (1966) *Nature (London)* **21,** 400–401.
18. Zornetzer, S. F., Thompson, R. & Rogers, J. (1982) *Behav. Neural. Biol.* **36,** 49–60.
19. Sarter, M., Bodewitz, G. & Stephens, D. N. (1988) *Psychopharmacology* **94,** 491–495.
20. Higgins, L. S., Rodems, J. M., Catalano, R., Quon, D. & Cordell, B. (1995) *Proc. Natl. Acad. Sci.*, in press.
21. Yamaguchi, F., Richards, S. J., Beyreuther, K., Salbaus, M., Carlson, G. A. & Dunnett, S. B. (1991) *NeuroReport* **2,** 781–784.
22. Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., *et al.* (1995) *Nature (London)* **373,** 523–527.

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Neurobiology

Early Alzheimer disease-like histopathology increases in frequency with age in mice transgenic for β-APP751

(Alz50/β-amyloid/β-amyloid precursor/Down syndrome)

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ABSTRACT β-Amyloid deposition and neurofibrillary tangle formation are two histopathological features of Alzheimer disease. We have previously reported that β-amyloid immunoreactive deposits form in the brains of transgenic mice programmed for neuronal expression of the 751-amino acid isoform of human β-amyloid precursor protein (β-APP751) and now describe that these animals also display Alz50 intraneuronal immunoreactivity similar to that seen in early Alzheimer disease. This suggests that abnormal β-APP expression and/or β-amyloid deposition promotes pathogenic alterations in tau protein. The frequency of both β-amyloid deposition and Alz50-positive neurons was twice as prevalent in brains from old (22 months) as compared to young (2–3 months) β-APP751 transgenic mice. This increase in histopathology with age in β-APP751 transgenic mice parallels the time-dependent progression seen in the human disease.

Alzheimer disease (AD) results in profound pathological changes in the brains of its victims. Two major histological characteristics of the disease are amyloid plaques and neurofibrillary tangles (NFTs). Identical structures are observed in brain tissues of individuals with trisomy 21, or Down syndrome (DS)—all of whom invariably develop AD (1, 2). Neuropathological changes thought to be the precursors to NFTs and amyloid plaques are apparent in DS by the second decade of life, providing a glimpse of early AD development (3–6).

AD plaques contain β-amyloid, an ≈4-kDa protein derived from a precursor, β-APP (β-amyloid precursor protein) (7). The proteolytic mechanism(s) by which β-amyloid is generated from β-APP is unclear. Once the β-amyloid peptide is produced, its development into a mature plaque is believed to progress from an amorphous to a highly ordered polymerized fibrillar structure. This putative plaque progression is based on studies examining DS brains spanning ages from fetus to adult (4–6). The highly polymerized β-amyloid deposits in both DS and AD have associated abnormal neuritic structures, including NFTs, dystrophic neurites, and neuropil threads (2).

While the biochemical composition of NFTs is not completely determined, the primary component thus far identified is tau protein, a cytoskeletal protein that associates with microtubules (8, 9). NFT tau protein (referred to as A68 or tau^{PHF}) is abnormally phosphorylated (10–12). Highly specific antibodies have been raised that selectively detect abnormal tau protein and stain NFTs and neuropil threads in AD brain but that do not stain normal adult brain. One such antibody that has been widely used for histopathological studies is Alz50 (13). Sparsely distributed neurons staining with Alz50 are observed in young adult DS brain (3). This Alz50 staining, which includes the cell soma, axons, and dendrites, indicates that tau in these neurons is also aberrant in its subcellular localization, as tau is normally confined to axons (9, 14). In older DS brains, the Alz50 immunoreactivity is indistinguishable from the pattern observed in AD (3). The relationship between amyloid formation and pathogenic alterations in tau is not clear.

An understanding of these pathological events in AD has been hampered by the lack of a well-characterized small animal model. We have generated transgenic mice that are programmed for neuronal expression of human 751-kDa β-APP (β-APP751) and that develop β-amyloid immunoreactive deposits in their brains (15, 16). Significantly, these transgenic mice show age-dependent learning and memory impairment in the relative absence of general behavioral or other neurological deficits (17). Here we describe the frequency and the sex and age relationship of β-amyloid deposits in brains of mice from multiple β-APP751 pedigrees. In addition, we have analyzed these same parameters in transgenic mice with regard to Alz50 immunoreactivity.

MATERIALS AND METHODS

RNA Expression in Transgenic Lines. Generation of mice expressing the human cDNA for β-APP751 from the neuron-specific enolase (NSE) promoter has been reported (15). Semiquantitative PCR amplification was carried out essentially according to the procedure of Golde *et al.* (18). Total RNA was isolated from individual or pooled brains and reverse transcribed using oligo(dT_{12-18}). Primers flank the KPI-domain coding region and correspond to the human sequence; the forward primer (5′-CACCACAGAGTCTGTGGAAG-3′) differs in two positions and the reverse primer (5′-AGGTGTCTCGAGATACTTGT-3′) differs in three positions from the murine sequence. Our amplification reaction was determined to be linear for 30 cycles. Amplified products were separated by electrophoresis on 8% polyacrylamide gels and visualized by autoradiography. Quantitation of radioactivity in amplified product bands was made by densitometric tracing of the autoradiogram with an Applied Image Lynx 4000 densitometer (Santa Clara, CA).

Immunohistochemistry. Preparation of all 6-μm midline coronal brain sections and immunohistochemistry were performed as described (15, 16), except that most brains were perfused with saline followed by 4% paraformaldehyde prior to removal. Alz50 was used at a 1:25 dilution in 5% nonfat dry milk for an overnight incubation at 4°C. Sections were counterstained with hematoxylin. Omission of the primary antibody was used to assess specificity of Alz50 immunoreactivity. Human AD brain tissue was from patients with clinically diagnosed AD.

RESULTS

Transgenic RNA Expression. β-Amyloid immunoreactive deposition occurring in brains of mice carrying a transgene of

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Abbreviations: β-APP, β-amyloid precursor protein; AD, Alzheimer disease; DS, Down syndrome; NFT, neurofibrillary tangle; NSE, neuron-specific enolase.

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human β-APP751 cDNA driven by the NSE promoter (NSE:β-APP751) has been qualitatively described (15, 16). The β-APP751 isoform was initially chosen for study in consideration of published data indicating that β-APP751 mRNA is elevated in AD (19, 20). Southern blots were used to determine the hemi- and homozygous state for NSE:β-APP751 animals. Six NSE:β-APP751 pedigrees [four of which have been described in part (15)] and wild-type mice of the parental strain constituted the set of animals used in this study. As an additional control set for comparison to NSE:β-APP751 mice, brain sections were also examined from animals generated from the same strain of mouse but transgenic for one of two distinct constructs driven by the NSE promoter.

Relative levels of transgenic β-APP expression were compared for all pedigrees by semiquantitative PCR (Fig. 1*A*). Primers flanking the KPI coding sequence (with one radiophospholabeled) were selected for limited PCR amplification (18). Template cDNA was prepared for amplification by reverse transcription of RNA isolated from pooled brain homogenates from three homozygous animals of a pedigree. The products of the PCR generated an 87-bp product derived from endogenous β-APP695 mRNA, which could readily be differentiated from the 255-bp product from β-APP751 mRNA. As reported by others (21–23), β-APP695 predominates in wild-type murine brain, while β-APP751 expression is minimal (Fig. 1 *A* and *B*). The results revealed that transgenic mRNA expression was greater than that of endogenous expression and that expression levels varied between pedigrees. When the PCR was allowed to proceed for an additional five cycles to increase sensitivity of the assay, the presence of β-APP751 mRNA was confirmed in all NSE:β-APP751 pedigrees and was greater than endogenous β-APP751 mRNA (Fig. 1*B*). The presence of transgenic transcription was unequivocally confirmed in all samples by using a second set of primers that amplify only accurately spliced NSE:β-APP chimeric mRNA from the transgene (15). To ensure that different expression levels were representative of variability between pedigrees and not reflecting differences in individuals used in the pooled brain RNA, we compared levels of four littermates from a single pedigree to the level of that pedigree's pool (Fig. 1*C*). The semiquantitative PCR result indicates both that pooled RNA was representative of the pedigree and that expression levels were constant among individual animals.

β-Amyloid Immunoreactive Deposits in Transgenic Mice. All NSE:β-APP751 pedigrees, as well as wild-type mice and mice transgenic for other constructs, were surveyed for the production of β-amyloid immunoreactive deposits in their brains. A monoclonal antibody, mAb4.1, was used for the immunohistochemical analysis. This antibody is highly selective for human over murine β-amyloid (16). The same antibody stains plaques in AD and DS brain and was used to reveal preamyloid deposits in NSE:β-APP751 transgenic mouse brains (15, 16). The profile obtained from this characterization most closely resembled that of the diffuse deposits observed in the brains of young DS adults. To determine the frequency of β-amyloid immunoreactive deposits, brain sections from a large number of animals from all three groups of mice (total, $n = 80$; β-APP751, $n = 33$; other transgenic, $n = 29$; wild type, $n = 18$) were examined. Animals well matched for age, sex, and transgene hemi- or homozygosity from each of the three groups were selected for the study and each transgenic pedigree was represented. At least three brain sections from each animal, and often more, were included in the study. A section was scored as positive if it contained one or more extracellular mAb4.1 immunoreactive deposits.

Quantifying the immunohistochemical results in this manner revealed that 27% of the brain sections from NSE:β-APP751 animals contained deposits and that this proportion was significantly greater than that measured for control transgenic or wild-type animals (Table 1). Immunoreactive deposits were detected in 22 of the 33 NSE:β-APP751 animals (67%) used in the study, including members of all six NSE:β-APP751 pedigrees. The proportion of brains containing deposits is likely to be greater than detected since only a small portion of the brain was sampled. Nevertheless, the overall frequency of diffuse deposits in the β-APP751 transgenic brain is low. Deposits that were observed, albeit rarely, in wild-type brains are likely to represent artifacts that cannot be distinguished from specific staining since the antibody has little affinity for murine β-amyloid (16). Immunoreactive deposits were never observed in multiple sections from the same control mouse brain: for some animals, up to 17 sections from a single brain were examined. In contrast, multiple positive sections from

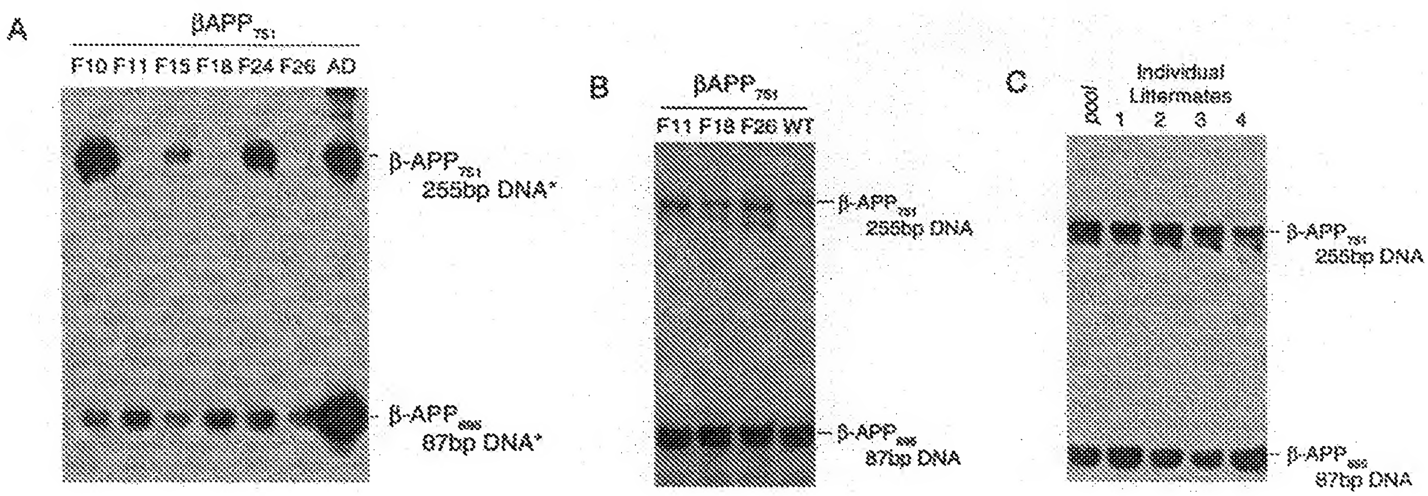

FIG. 1. β-APP mRNA expression in transgenic pedigrees. (*A*) Comparative mRNA expression analysis between NSE:β-APP751 pedigrees by semiquantitative PCR. β-APP695 (87 bp) and β-APP751 (255 bp) ^{32}P-labeled amplification products were produced by limited PCR from reverse-transcribed mRNA, separated by gel electrophoresis, and autoradiographed. Individual samples represent mRNA derived from three pooled brains of a pedigree propagated from each founder. Individual animals derived from each NSE:β-APP751 founder (F10, F11, F15, F18, F24, and F26) and wild type (WT) are designated. WT and AD indicate PCR products using mRNA isolated from wild-type mouse brains and AD frontal cortex, respectively. (*B*) Additional cycles of amplification of β-APP751 mRNA from low level expression pedigrees. (*C*) Comparative mRNA expression analysis within a transgenic pedigree. A limited PCR experiment identical to that in *B* was carried out with mRNA prepared from individual brains of four littermates (samples 1–4) and on mRNA from three pooled brains (pool). All brains were derived from the β-APP751 F11 pedigree.

Table 1. Frequency of β-amyloid immunoreactive deposits in brains from transgenic and wild-type mice

	Sections positive, %	No. of sections
NSE:β-APP751	27*†	122
Control transgenic	2	94
Wild type	5	107

NSE:β-APP751 set included 33 animals from six independent pedigrees; control transgenic set included 20 animals from four pedigrees bearing one construct and 9 animals from three pedigrees bearing a second construct; 18 wild-type mice were included from the strain used to create the transgenic pedigrees. Three or more midline coronal sections were stained from each animal. Three investigators independently scored the sections with close agreement.
*Significantly different from value for control transgenic; $P < 0.01$ (two tailed).
†Significantly different from value for wild type; $P < 0.01$ (two tailed).

NSE:β-APP751 brains were seen. Thus, the scoring of small diffuse deposits may include a 2–5% background.

Data from the 33 NSE:β-APP751 animals used in this study were subdivided by sex and transgene hemi- or homozygosity. Comparing the frequency of deposits in male and female mice revealed no difference. However, animals homozygous for the transgene displayed twice the frequency of β-amyloid immunoreactive deposits in their brains compared to hemizygous animals (Table 2).

Alz50 Immunoreactivity. Aberrant tau isoforms, A68 being the largest, of the paired helical filaments comprising NFTs of AD brain can be detected by the monoclonal antibody Alz50 (13). A68 is detected by Alz50 in normal fetal development but is not observed in adult human brain except in dementing neurodegenerative disorders such as AD. Although Alz50 detects normal as well as abnormal forms of human tau on Western blots (24), it shows little immunohistochemical staining of normal human brain (25). In contrast, Alz50 immunohistochemical staining of AD brain reveals pathological structures including NFTs, dystrophic neurites, and neuropil threads (13). To determine whether AD-like cytoskeletal perturbations in tau are detectable in the transgenic mice, we immunostained brain sections with Alz50. This antibody was selected over others raised to human aberrant tau since to date only Alz50 has been shown capable of recognizing tau from rodents (26).

Numerous neuropil threads and neurofibrillary tangles are revealed by Alz50 immunoreactivity in AD brain tissue (Fig. 2A). Alz50-stained brain sections from a young DS adult show immunoreactive neurons and processes of a different quality (Fig. 2 B and C). The reactive neurons are sparsely distributed in the section, as has been observed by others (13), and staining is punctate rather than of the dense fibrillar nature of mature NFT. NSE:β-APP751 brain sections stained with Alz50 also revealed immunoreactive neurons, processes, as well as fields of puncta in the neuropil. Two examples of such staining from transgenic mouse entorhinal cortex are shown at low magnification in Fig. 2 D and E for comparison with the human AD and DS examples. Higher magnification illustrations of abnormal immunoreactivity with neurons, processes, and puncta from NSE:β-APP751 mouse are shown in Fig. 3. Alz50-positive structures were frequently found in clusters but solitary stained cells or processes were also seen. Generally, only a few Alz50-positive cells were observed in a cluster. Stained neuronal soma in the NSE:β-APP751 mice were exclusively localized to the cerebral cortex (both deep and superficial layers), the thalamus, and the amygdala. Immunoreactive processes were frequently noted in these same brain regions and occasionally in the hippocampus. As in the case of β-amyloid immunoreactivity, Alz50 staining of NSE:β-APP751 mouse brains most closely resembled staining in the young adult DS brain and not the late-stage AD brain.

Table 2. Frequency of β-amyloid immunoreactive deposits in brains from NSE:β-APP751 transgenic mice

NSE:β-APP751 group	Sections positive for β-amyloid immunoreactive deposits, % (no. of sections)
Sex	
♀	27 (63)
♂	25 (59)
Genotype	
AA	31 (80)*
Aa	17 (42)

NSE:β-APP751 set and scoring are described in Table 1. *AA*, homozygous for transgene; *Aa*, hemizygous for transgene.
*Significantly different from value for *Aa*; $P < 0.01$ (one tailed).

Alz50 immunoreactivity has been described in normal adult rodent brain in which staining of periventricular structures, including the median eminence and ventromedial hypothalamus, is found (26). In the wild-type adult mouse brain used in these experiments, this normal Alz50 staining was composed solely of punctate neuropil and served as a positive control for the procedure. We also observed occasional staining of puncta in the internal capsule and thalamic habenula of wild-type mice.

A comparative survey of Alz50 immunoreactivity was carried out with brain sections from the NSE:β-APP751, wild-type, and control transgenic groups. Abnormal Alz50 reactivity was defined in this study as the presence of stained neurons, regardless of location, as well as puncta in the neuropil of the cortex, thalamus (excluding the habenula and periventricular regions), and/or hippocampus. Abnormal Alz50 immunoreactivity was found in about half of the brain sections from NSE:β-APP751 mice (Table 3) with an equal distribution between male and female mice. The sections were taken from brains of 15 animals derived from three NSE:β-APP751 pedigrees. A minimum of three slides per animal were examined and at least one slide scored positive from 11 of the 15 animals (73%). As is the case for β-amyloid deposits, the absolute percentage of brains bearing abnormal Alz50 immunoreactive structures is probably higher than revealed by our limited (18 μm) sample. Alz50 immunoreactivity was relatively rare in brain sections from 12 wild-type mice and from 15 control transgenic mice (Table 3). The Alz50 immunoreactive structures (filled cells, puncta, and tracts) were present in the same anatomical areas of the NSE:β-APP751 brain as the β-amyloid deposits, such as the cortex, hippocampus, and thalamus. Colocalization of these two staining entities has been observed in the NSE:β-APP751 mouse brain (16) but has not been extensively examined. While bulbous dystrophic neurites containing tau^{PHF} are found in association with mature plaques in AD brain, the neural soma giving rise to these terminals may be distant. In the transgenic mouse abnormal Alz50 immunoreactive structures were most common in the amygdala, followed by the cortex, thalamus, and hippocampus, while β-amyloid immunoreactive deposits were most common in the cortex and hippocampus, followed by the thalamus. It is noteworthy that the NSE:β-APP751 animals that scored positive for β-amyloid immunoreactivity were generally positive for Alz50 immunoreactivity and those that were negative for β-amyloid were negative for Alz50 (79% concordance). Again, given the limited proportion of the brain sampled, "positive" and "negative" assessments are a more accurate reflection of the density of such structures than of their presence versus absence.

Frequency of β-Amyloid and Alz50 Immunoreactive Structures Increases with Age. We were interested in determining whether the histopathology in these animals also increased with age. In the previous study surveying pedigrees, a trend toward increased frequencies of both features with age was seen in NSE:β-APP751 mice. However, the animals used were a random collection of ages, hemi- and homozygotes for the

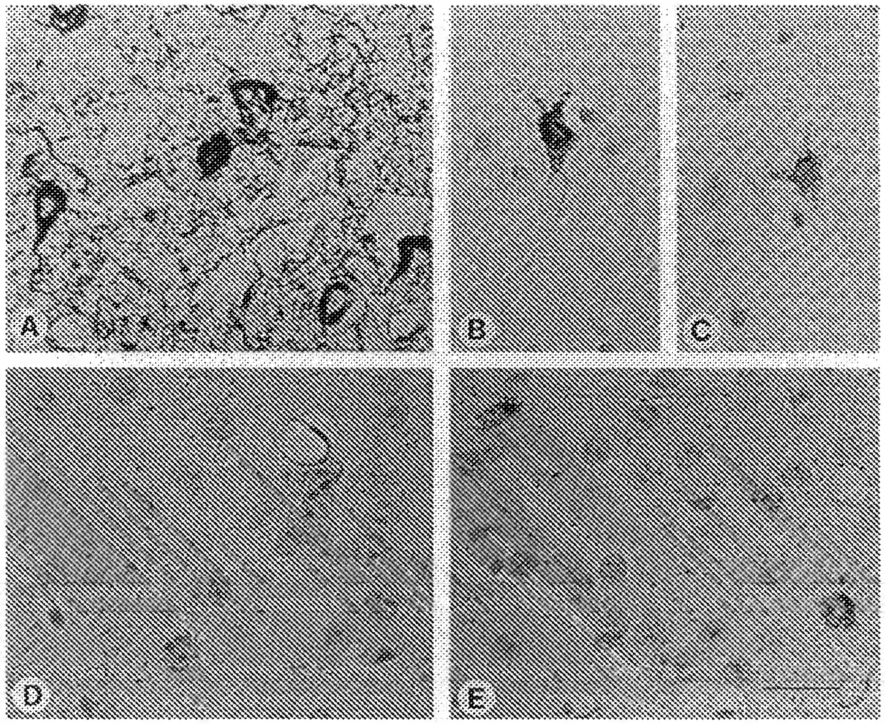

FIG. 2. Alz50 immunohistochemistry of human and transgenic mouse brain. (*A*) Human AD frontal cortex displaying classical NFT and neuropil thread pathology. (*B* and *C*) Human 16-year-old male DS frontal cortex showing an infrequently stained neuron, both soma, and processes. (*D* and *E*) NSE:β-APP751 transgenic mouse brain, entorhinal cortex. ($\times$50.) (Bar = 50 μm.)

transgene, and pedigrees, and thus too few matched animals were present for rigorous analysis. Hence, we selected a single pedigree, NSE:β-APP751 F10, for which we had a large number of young (2–3 months; n = 12) and old (22 months; n = 12) homozygous mice to examine. The study, in which investigators scoring the brain sections were blind to the age of animals, revealed that the frequency of β-amyloid immunoreactive deposits increased with age: nearly twice the frequency in the old set of animals compared with that in the young set was observed (Table 4). Similarly, Alz50 immunoreactive structures occurred at twice the frequency in the older population of NSE:β-APP751 mice (Table 4). The concordance of animals for which the portion of brain sampled was positive or negative for both β-amyloid and Alz50 markers was 75%. This value is in close agreement (75% vs. 79%) with the value obtained from our first survey.

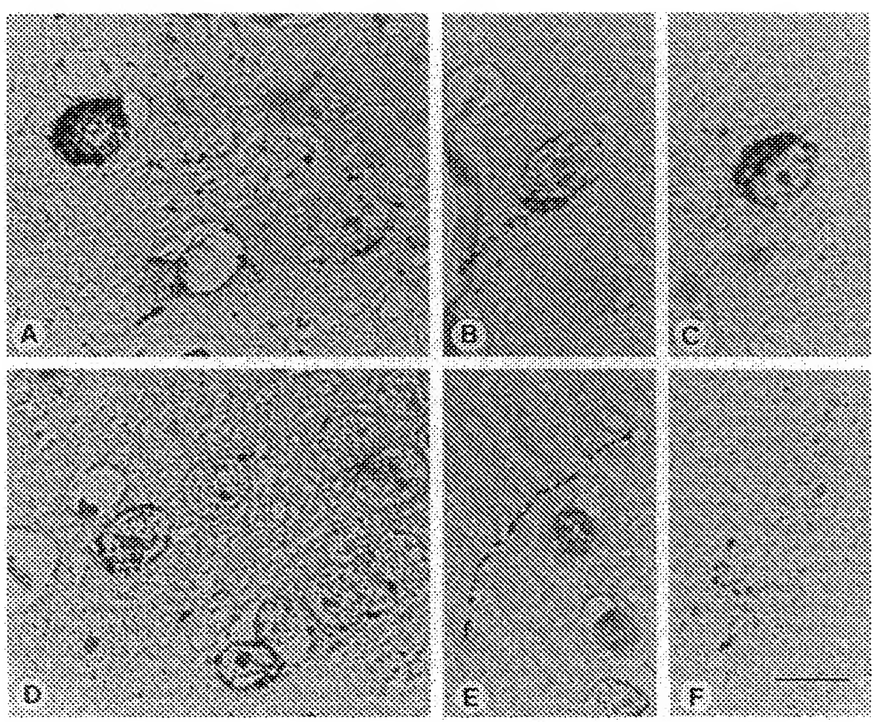

FIG. 3. Alz50 immunohistochemistry of transgenic mouse brain. (*A–D*) Stained cortical neurons. (*E*) Stained neuronal process in hippocampal molecular layer. (*F*) Cortical field of immunoreactive puncta. ($\times$130.) (Bar = 20 μm.)

Table 3. Frequency of abnormal Alz50 immunoreactive structures in mouse brain

	Sections positive, %	No. of sections
NSE:β-APP751	42*†	52
Control transgenic	2	45
Wild type	6	53

NSE:β-APP751 set included 15 animals representing three independent pedigrees; control transgenic set included 10 animals from three independent pedigrees bearing one construct and 5 animals from three pedigrees bearing a second construct; wild type included 12 animals. Three or more midline coronal sections were immunostained from each animal. Sections were scored independently by three investigators with close agreement.

*Significantly different from value for control transgenic; $P < 0.01$ (two tailed).

†Significantly different from value for wild type; $P < 0.01$ (two tailed).

DISCUSSION

β-Amyloid deposition and NFT formation are histopathological hallmarks of AD. Here we describe transgenic mice that display the apparent precursors of these two pathological structures. Both β-amyloid deposits (16) and Alz50 reactive neurons observed in the β-APP751 transgenic mouse brain resemble the presenile structures observed in the early AD pathology of the young adult DS brain. That subtle increases in neuronal expression of human β-APP751 may be critical to establishing a pathogenic state is further substantiated by our data in which mice homozygous for the β-APP751 transgene have twice the frequency of β-amyloid immunoreactive deposits as their hemizygotic counterparts.

We have also demonstrated that β-APP751 overexpression and/or β-amyloid formation can lead to cytoskeletal pathology as defined by Alz50 immunoreactivity. Abnormal tau, which is detected by Alz50, occurs in a variety of dementing disorders and may be part of a common neurodegenerative pathway following various initial insults. That preamyloid promotes abnormalities in tau is consistent with observations of DS pathology in which preamyloid formation is seen to precede NFT formation (4). The distribution of Alz50 immunoreactive neurons and of β-amyloid immunoreactive deposition in β-APP751 transgenic mice resembles that in AD.

Last, we have found that the histopathology thus far characterized in the transgenic mice increases with age. The age-related development of histopathology correlates with the development of a cognitive phenotype analogous to deficits in early AD: NSE:β-APP751 mice show specific and age-dependent impairment of learning and memory in the absence of general behavioral or neurological perturbations (17). It is noteworthy that mice with relatively subtle histopathology manifest behavioral deficits. This observation may be due to lesser ability of mice to compensate compared to humans, or it may reflect effects of human β-APP751 neuronal overexpression other than the histopathological features examined here. Whether a relationship exists of β-amyloid immunoreactive deposits and abnormal Alz50 immunoreactive structures to learning and memory impairment in the transgenic mice will be of interest to be determined.

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Table 4. Frequency of histopathological structures increases with age in NSD:β-APP751 F10 mouse brain

Age, months	Sections positive (no. of sections)	
	β-Amyloid deposits	Abnormal Alz50
2–3	29% (35)	33% (36)
22	49% (35)	69% (36)

Twelve mice in each age group, all homozygous for the transgene and all from the NSE:βAPP751 F10 pidigree, were included in the study. Three midline coronal sections were strained for β-amyloid immunoreactivity and three were stained for Alz50 immunoreactivity per animal and then scored by two investigators blind to the identity of the sections. Difference in values for old and young groups is significant ($P < 0.05$; one tailed).

1. Burger, P. C. & Vogel, F. C. (1973) *Am. J. Pathol.* **73,** 457–476.
2. Mann, D. M. A. (1988) *Mech. Ageing Dev.* **43,** 99–136.
3. Wolozin, B., Scicitella, A. & Davies, P. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6202–6206.
4. Giaccone, G., Tagliavini, F., Linoli, G., Bouras, C., Frigerio, L., Frangione, B. & Bugiani, O. (1989) *Neurosci. Lett.* **97,** 232–238.
5. Mann, D. M. A. & Esiri, M. M. (1989) *J. Neurol. Sci.* **89,** 169–179.
6. Motte, J. & Williams, R. S. (1989) *Acta Neuropathol.* **77,** 535–546.
7. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Greshik, K.-H., Multhaup, G., Beyreuther, K. & Muller-Hill, B. (1987) *Nature (London)* **325,** 733–736.
8. Goedert, M., Wischik, C. M., Crowther, R. A., Walker, J. E. & Klug, A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4051–4055.
9. Kosik, K. S., Orecchio, L. D., Binder, L., Trojanowski, J. Q., Lee, V. M.-Y. & Lee, G. (1988) *Neuron* **1,** 817–825.
10. Greenberg, S. G. & Davies, P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5827–5831.
11. Lee, V. M.-Y., Balin, B. L., Otvos, K. & Trojanowski, J. Q. (1991) *Science* **251,** 675–678.
12. Goedert, M., Spillantini, M. G., Cairns, N. J. & Crowther, R. A. (1992) *Neuron* **8,** 159–168.
13. Wolozin, B. L., Pruchnicke, A., Dickson, D. W. & Davies, P. (1986) *Science* **232,** 648–650.
14. Binder, L. I., Frankfurter, A. & Rebhun, L. I. (1985) *J. Cell Biol.* **101,** 1371–1378.
15. Quon, D., Wang, Y., Catalano, R., Scardina, J. M., Murakami, K. & Cordell, B. (1991) *Nature (London)* **352,** 239–241.
16. Higgins, L. S., Holtzman, D. M., Rabin, J., Mobley, W. C. & Cordell, B. (1994) *Ann. Neurol.* **35,** 598–607.
17. Moran, P. M., Higgins, L. S., Cordell, B. & Moser, P. C. (1994) *Neurobiol. Aging* **15,** Suppl. 1, 48 (abstr.).
18. Golde, T. E., Estus, S., Usiak, M., Younkin, L. H. & Younkin, S. G. (1990) *Neuron* **4,** 253–267.
19. Neve, R. L., Finch, E. A. & Dawes, L. P. (1988) *Neuron* **1,** 669–677.
20. Johnson, S. A., McNeill, T., Cordell, B. & Finch, C. E. (1990) *Science* **248,** 854–857.
21. Anderson, J. P., Refolo, L. M., Wallace, W., Mehta, P., Krisnamurthi, M., Gotlib, J., Bierer, L., Haroutunian, V., Perl, D. & Robakis, N. K. (1989) *EMBO J.* **8,** 3627–3632.
22. Schubert, W., Prior, R., Weidemann, Dircksen, H., Multhaup, G., Masters, C. L. & Beyreuther, K. (1991) *Brain Res.* **563,** 184–194.
23. Potempska, A., Styles, J., Mehta, P., Kim, K. S. & Miller, D. L. (1991) *J. Biol. Chem.* **266,** 8464–8469.
24. Ksiezak-Reding, H. & Yen, S.-H. (1987) *J. Neurochem.* **48,** 455–462.
25. Wolozin, B. L. & Davies, P. (1987) *Ann. Neurol.* **22,** 521–526.
26. Byne, W., Mattiace, L., Kress, Y. & Davies, P. (1991) *J. Comp. Neurol.* **306,** 602–612.